BMP6 in Renal Cell Carcinoma Promotes Tumor Proliferation through IL-10-dependent M2 Polarization of Tumor-Associated Macrophages*

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*This work was supported in part by the Tanzman Foundation, Mr. John Shein, and Mr. Malcolm Wernik.

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Running title: BMP-6 induces M2 polarization of macrophages in RCC

Conflict of Interest Disclosure: none
ABSTRACT

Dysregulated bone morphogenetic proteins (BMPs) may contribute to the development and progression of renal cell carcinoma (RCC). Herein, we report that BMP-6 promotes the growth of RCC by IL-10-mediated M2 polarization of tumor-associated macrophages (TAMs). BMP-6-mediated IL-10 expression in macrophages required Smad5 and STAT3. In human RCC specimens, the three-marker signature BMP-6/IL-10/CD68 was associated with a poor prognosis. Furthermore, patients with elevated IL-10 serum levels had worse outcome after surgery. Together our results suggest that BMP-6/macrophage/IL-10 regulates M2 polarization of TAMs in RCC.
INTRODUCTION
Kidney cancer is the seventh most common cancer with an estimated 58,240 new cases and 13,040 deaths in 2010 (1). Histologically, the most common type of kidney cancer is clear cell renal cell carcinoma (RCC) (80-85%) (2). As with most solid malignancies, localized RCC are usually cured with surgery. However, approximately 30% of patients with clinically localized RCC eventually recur after resection.

Bone morphogenetic proteins (BMPs) comprise the largest family within the transforming growth factor-beta (TGF-β) superfamily. Although originally reported as factors that induce bone and cartilage formation (3), BMPs have been shown to be critical for development (4-6). More recently, we and others have reported that BMPs regulate the immune system as BMP-6 inhibits B and T cell proliferation (7, 8) and activates macrophages (9, 10). In the context of RCC, BMP-4, -6, and -7 are often overexpressed (11, 12) while BMP antagonist Sclerostin domain–containing-1 (SOSTDC1) is down-regulated (13). At the same time, we have observed that human RCC cells frequently have a loss of expression of BMP receptors (11), suggesting a paracrine role for BMP-6 in RCC.

Macrophages are an essential component of the host defense system and have critical roles in both innate and adaptive immune responses (14). In solid malignancies, a large number of macrophages is usually found infiltrating the tumor. These tumor-associated macrophages (TAMs) (15, 16) are recruited from the circulating peripheral blood monocytes by chemotactic factors and chemokines (17, 18). When tumor cells arise, macrophages are capable of mounting an anti-tumor response (19). However, the decision to mount an anti-tumor response is in part regulated by the balance of pro- and anti-inflammatory factors present within the tumor microenvironment (14, 20). Indeed, a body of evidence suggests that macrophages in tumors can promote cancer progression and metastasis by stimulating angiogenesis, tumor growth, and cellular migration and invasion (21).

One cytokine that has been suggested to play a role in regulating macrophages is interleukin-10 (IL-10), a type 2 cytokine. IL-10 is produced by monocytes, activated macrophages, and a subtype of dendritic cells (22). In cancers, additional sources of IL-10 include the alternatively activated M2 macrophages and TAMs (23, 24). Since, the major stimulus for the IL-10 expression is inflammation, IL-10 plays a major role in the negative feedback loop that prevents uncontrolled inflammation (25). In solid tumors, IL-10 has been
detected in a number of malignancies (26). Recently, it has been suggested that a local production of IL-10 results in a tumor microenvironment that favors cancer cell survival and metastases (23, 27). In RCC, IL-10 and IL-10 receptor expression has been correlated with increased incidence of metastasis (28). Nevertheless, the clinical significance of IL-10 and the mechanism of its induction in cancers remain largely unknown. In this study, we report that BMP-6 in murine RCC cell lines is pro-tumorigenic and involves IL-10-mediated M2 polarization of TAMs.

MATERIALS AND METHODS

Animal Studies
Balb/c and IL-10KO mice (Jackson Laboratory, Bar Harbor, ME) were inoculated subcutaneously with BMP-6/RenCa and BMP-6/RAG cells. For BMP-6 induction, drinking water containing 2 mg/ml of doxycycline (Sigma-Aldrich, St. Louis, MO) in 5% sucrose was used. For adoptive transfer of macrophages, 5x 10^6 cells were injected directly into tumors. Unless indicated, 5 animals were used per group.

Cell culture
RAW 264.7, RenCa, and RAG were purchased from the American Type Culture Collection (ATCC) (Manassas, VA) and routinely maintained in as recommended by the vendor. To obtain murine peritoneal macrophages (PMs), thioglycollate (Sigma-Aldrich, St. Louis, MO) was dissolved in dH2O and injected intraperitoneally into mice. Animals were sacrificed 3 days later and PMs were isolated from the peritoneal lavage and cultured as described (29). Primary human peripheral blood monocytes (hPBMC) were purchased from STEMCELL Technologies, Inc (Vancouver, Canada). The STAT3 inhibitor WP1066 (EMD Chemicals, Gibbstown, NJ) was used at 10 μg/ml. BMP-6 was obtained from R&D Systems (Minneapolis, MN).

BMP-6 inducible murine renal cell carcinoma cell lines
Human BMP-6 cDNA was cloned into the tetracycline inducible vector pLenti4/Dest (Invitrogen). Simultaneously, RenCa and RAG were infected sequentially with the Tetracycline Repressor (TR) virus followed by pLenti4/BMP-6 virus.

**RT-PCR**
Total RNA was extracted with Trizol (Invitrogen, Carlsbad, CA) and RT-PCR was carried out using the One-step SuperScript RT-PCR® kit (Invitrogen). Reverse transcription was completed as recommended. For all primers, the annealing temperature was 55°C. Primer sequences are shown in Supp Table 1.

**Immunoblot**
Cells were harvested using the Cell Lysis Buffer (Cell Signaling Technology, Danvers, MA). After centrifugation, electrophoresis was carried out with 30 μg of protein using 12% SDS-PAGE gel and analyzed using enhanced chemiluminescence (Thermo Scientific, Rockford, IL).

**Antibodies**
The following antibodies were used in this study: mouse IL-10 (VWR), human IL-10 (R&D Systems), β-actin (Sigma), human BMP-6 blocking antibody (R&D), human BMP-6 (Abcam), Myc (Sigma), mouse STAT3, mouse phospho STAT3 (Cell signaling), Smad 5 (Cell signaling), phospho Smad 5 (Cell signaling), Smad 1/5 (Santa Cruz), phospho Smad 1/5 (Cell signaling), mouse CD206 (Serotec), human CD206 (BD), mouse CD163 (Santa Cruz), human CD163 (Abcam), human GAPDH (Santa Cruz), and human ki67 (Abcam).

**Luciferase assay**
Luciferase assay was performed using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI) per manufacturer’s protocol. Cells were transfected using lipofectamine 2000 (Invitrogen, Carlsbad, CA). All experiments were repeated at least 3 times.

**Transient transfection of BMP receptors**
RAW 264.7 cells were plated onto 6-well plates and lipofectamine 2000 (Invitrogen) at 1 μl/ml was used to transfect 1 μg/ml of plasmids.
RNA knock-down studies

Sequences of shRNA and construction of expression system have previously been published (9, 29). Knock-down of STAT3 and STAT4 was carried out using siRNA (Qiagen).

Chromatin Immunoprecipitation (ChIP) Assay

EZ-ChIP kit (Millipore) was used. Cells were harvested, fixed, and incubated with the indicated antibodies. Then DNA was sonicated and purified with spin column and analyzed with PCR.

Immunoprecipitation and Immunoblot

NE-PER (Thermo Scientific, Rockford, IL) was used to separate cytoplasmic and nuclear proteins. After pre-clearing with protein A sepharose (GE healthcare), indicated primary antibody was added to the lysate and incubated at 4°C. Antibody was pulled down with protein A sepharose and washed with ice-cold lysis buffer and proteins were analyzed by immunoblot analysis.

ELISA

IL-10 and BMP-6 levels in the culture media and serum were measured using an ELISA kit (R&D Systems). The indicated values represent the average of 4 separate measurements.

Clodronate Liposome

Clodronate- and PBS-loaded liposomes were kindly provided by Clodronate Liposomes.org (Amsterdam, Netherlands). Mice were injected with 0.2 ml of either clodronate-liposomes (final dose of 25 mg/kg) or PBS-liposomes every 4 days intraperitoneally or intratumorally.

Human RCC tissues, serum, immunohistochemistry, and confocal microscopy

Seventy-four formalin-fixed paraffin-embedded human RCC tissues were obtained from Chungbuk National University (Cheongju, Korea) while human clear cell RCC tissue array containing 50 cases were purchased from Imgenex (San Diego, CA). Serum samples were available in 56 of the patients from Chungbuk National University. The sections were processed
using a routine laboratory procedure and counterstained with hematoxylin. The results were interpreted by 2 independent investigators who were blinded to each other’s reading.

Statistical analysis
Student’s $t$ test and Pearson correlation were performed. Clinical data were evaluated using Kaplan-Meier and multivariate Cox analysis. $P$-value $<0.05$ was considered statistically significant.

RESULTS
BMP-6 is pro-tumorigenic in RCC cells
Since published studies have suggested the dysregulation of BMPs in RCC (11, 13), tetracycline inducible human BMP-6 expression system based on lentivirus was established in a murine RCC cell line, RenCa. Human BMP-6 cDNA was chosen to track the expression of exogenous BMP-6. Previously, the use of human BMP-6 in mouse has been validated (9, 29). Because RenCa cells were originally derived from Balb/c mice, syngeneic tumors can be established in immune-competent animals. A representative result demonstrating the inducibility of BMP-6 mRNA and protein by tetracycline is shown in Fig 1A and 1B. One clone with a relatively high inducible level of BMP-6 was designated as RenCa/BMP-6. When BMP-6 expression was induced in RenCa/BMP-6 in tissue culture (Tet$^+$), no significant effect on cellular proliferation was observed (Fig 1C). However, in Balb/c mice, the induction of BMP-6 (doxy$^+$) demonstrated a significantly faster tumor growth without a noticeable change in histopathology (Fig 1D). This pro-tumorigenic effect of BMP-6 only in vivo implicates a paracrine mechanism. Along this line, it has recently been reported that BMPs regulate immune effector cells including macrophages (9, 10). To investigate whether macrophages mediate the pro-tumorigenic effect of BMP-6 in RCC, clodronate-liposome was used to selectively remove macrophages (30). The efficacy of clodronate-liposome in removing macrophages was confirmed in RenCa/BMP-6 syngeneic tumor model (Supp Fig 1A and B). As predicted, BMP-6’s pro-tumorigenic effect was completely blocked with clodronate-liposome (Fig 1E). In these tumors, clodronate-liposome had no effect on BMP-6 induction by doxycycline (Supp Fig 1C).

Next, the identical tetracycline-inducible BMP-6 system was developed in another murine kidney cancer cell line, RAG. The best clone with inducible BMP-6 expression was
designated RAG/BMP-6. When RAG/BMP-6 was inoculated subcutaneously into Balb/c mice, induction of BMP-6 (doxy+) significantly enhanced tumor growth (Supp Fig 2). Furthermore, clodronate-liposome injection reversed the oncogenic effect of BMP-6. These results collectively suggest that BMP-6’s pro-tumorigenic activity requires macrophages.

**Macrophages mediate the pro-tumorigenic effect of BMP-6 via IL-10**

When the murine macrophage cell line RAW 264.7 was treated with BMP-6, multiplex PCR revealed that BMP-6 upregulated the expression of a number of cytokines including IL-10. When RAW 264.7, murine peritoneal macrophages (PMs), THP-1 (human monocyte cell line), and human peripheral blood monocytes (hPBMC) were treated with BMP-6, IL-10 mRNA and protein were induced in a concentration- and time-dependent manner (Fig 2A and B); quantitative RT-PCR in RAW 264.7 is shown in Supp Fig 3A. IL-10 mRNA induction by BMP-6 was detected as early as 1 hour and peaked at 24 hours in PMs. ELISA revealed that the concentration of IL-10 in the conditioned media exceeded 120 pg/ml when RAW 264.7 was treated with 100 ng/ml BMP-6 (Supp Fig 3B). This level of secreted IL-10 was comparable to that when macrophages were stimulated with lipopolysaccharide (LPS).

To evaluate the role of IL-10 in vivo, IL-10 knockout (IL-10KO) mice were used. After inoculation, BMP-6 expression was induced (doxy+) when tumors became palpable. The results surprisingly demonstrated not only a retardation of tumor growth but also a complete elimination of tumors within 4 weeks after the induction of BMP-6 (Fig. 2C). When these mice were rechallenged with RenCa/BMP-6 and RenCa, no tumors developed (data not shown). To examine the histopathological changes of the tumors during regression in IL-10KO mice, doxycycline administration was started when the tumor size reached 5 mm in diameter. All tumors were harvested two weeks later. Hematoxylin staining demonstrated a dramatic increase in neutrophil infiltration and extensive tumor necrosis in tumors expressing BMP-6 (Fig 2D). These observations suggest that BMP-6 induces local inflammation and tumor rejection in the absence of IL-10.

**BMP-6 induces IL-10 expression in macrophages via BMP-RII and ALK2/3**

To determine the mechanism of IL-10 induction by BMP-6, we used the transcription inhibitor actinomycin D (ActD) and the translation inhibitor cycloheximide (CHX). IL-10 induction was
blocked only by ActD in both RAW 264.7 and PMs (Fig 3A). In order to expedite the dissection of the signaling pathway involved in BMP-6-induced IL-10 expression, we next constructed a luciferase reporter vector containing the IL-10 promoter (IL10-Luc). When IL10-Luc was transiently transfected into RAW 264.7, BMP-6 increased luciferase activity approximately 2.5-fold within 6 hrs (Supp Fig 3C).

BMPs signal through a heteromeric complex of types I and II receptors. When each of the three known BMP type II receptors were co-transfected with IL10-Luc into RAW 264.7, only BMP-RII overexpression increased IL-10 promoter activity upon treatment with BMP-6 (Fig 3B). Conversely, knock-down experiments using short hairpin RNA (shRNA) (9) demonstrated the suppression of luciferase activity only when BMP-RII was targeted (Fig 3C). To identify the functional BMP type I receptor(s), constitutively active mutant constructs were used. Macrophages express only two of the three known BMP type I receptors – ALK2 and 3 (9). Overexpression of constitutively active ALK2 and 3 (CA-ALK2 and CA-ALK3, respectively) increased the luciferase activity more than 10-fold (Fig 3D). When ALK2 and 3 were knocked down using shRNA (9), BMP-6 no longer induced luciferase activity (Fig 3E). These results collectively suggest that BMP-RII along with ALK2 and 3 are the functional BMP-6 receptors that mediate IL-10 induction in macrophages.

**IL-10 induction requires Smad5**

Following the activation of receptors, the canonical BMP signaling requires receptor-mediated Smads (R-Smads) (Smad1, 5, and 8) and co-Smad (Smad4). When the Smad-dependent pathway was blocked in RAW 264.7 with dominant negative Smad4 (Smad4DN), IL-10 induction by BMP-6 was completely blocked (Fig 4A). When each of the R-Smads was overexpressed in RAW 264.7, only Smad5 transfection increased luciferase activity upon treatment with BMP-6 (Fig 4B). This activation of IL-10 promoter by BMP-6 was then blocked when Smad5 was knocked down using shRNA (Fig 4C) (29). Serial deletion of IL-10 promoter subsequently demonstrated that the BMP-6 response element within IL-10 promoter is located between 588 and 800 bp 5’ to the transcription start site (Fig 4D). ChIP analysis revealed that Smad5 bound to the -588 to -800 region of IL-10 promoter (Fig. 4E).

**STAT3 is required for IL-10 induction**
Analysis of IL-10’s BMP-6 responsive region revealed no consensus Smad binding element (SBE). However, one STAT binding motif was identified. Previously, it has been reported that STAT5 interacts with Smad2 of the TGF-β signaling pathway (31). Of the known subtypes of STATs, STAT3 has been reported to regulate IL-10 expression (32). Accordingly, immunoblot was carried out to determine the phosphorylation status of STAT3 after treating RAW 264.7 with BMP-6. The results demonstrated increased phosphorylation of STAT3 (pSTAT3) within 30 min after BMP-6 treatment (Fig 5A). Blocking STAT3 with the small molecule inhibitor WP1066 (33) completely neutralized IL-10 induction by BMP-6 (Fig 5B). Next, studies using dominant negative STAT3 (STAT3DN) and small interfering RNA (siSTAT3) also demonstrated a complete abrogation of IL-10 induction by BMP-6 in RAW 264.7 (Fig 5C, Supp Fig 4A). When RAW 264.7 was transiently transfected with siSTAT3 and IL10-Luc, BMP-6 no longer increased luciferase activity (Supp Fig 4B). The complementary experiment in which STAT3 and IL10-Luc were overexpressed in RAW 264.7, BMP-6 increased the luciferase activity significantly (Fig 5D). Surprisingly, overexpression of STAT4 repeatedly blocked the activation of IL-10 promoter by BMP-6.

Next, ChIP assay demonstrated that STAT3 bound to the -588 to -800 bp region of IL-10 promoter (Fig. 5E). Since Smad5 also bound to the same region, we subsequently tested whether STAT3 interacts with Smad 5. When each of the myc-tagged R-Smads was expressed in RAW 264.7, immunoprecipitation against endogenous STAT3 pulled down Smad5 while immunoprecipitation against myc-Smad5 precipitated out STAT3 (Supp Fig 4C). The physical interaction between endogenous Smad5 and STAT3 in macrophages when stimulated by BMP-6 was then confirmed (Fig 5F).

Consistent with the known functions of Smad5 and STAT3, confocal immunofluorescence microscopy showed that both STAT3 and Smad5 translocate to the nucleus of RAW 264.7 when treated with BMP-6 (Supp Fig 4D). This BMP-6-induced nuclear translocation of STAT3 was still detected in RAW 264.7 even when Smad5 was knocked down (Supp Fig 4E). The reverse experiment revealed that the nuclear translocation of Smad5 still occurred when STAT3 was knocked down (Supp Fig 4F). Subsequently, co-immunoprecipitation of STAT3 and Smad5 was observed only in the nuclear fraction (Fig 5G). In the absence of exogenous BMP-6, low levels of STAT3 and Smad5 were co-immunoprecipitated out in RAW 264.7, likely suggesting a low basal level of expression of BMPs.
In RenCa/BMP-6 tumors, immunoblot confirmed the presence of both phosphorylated STAT3 and Smad1/5 (Fig 5H). Subsequently, immunofluorescence microscopy demonstrated the co-localization of pSTAT3, pSmad1/5, and F4/80 (murine macrophages) (Fig 5I). Interestingly, the induction of BMP-6 demonstrated a significantly higher number of macrophages within the tumors. These results confirm that both Smad5 and STAT3 are phosphorylated in macrophages upon BMP-6 induction in vivo.

**BMP-6 induces M2 macrophage polarization via IL-10**

IL-10 induces M2 polarization of macrophages that leads to local immunosuppression (19). When RAW 264.7 was co-cultured with RenCa, there was a ten-fold increase in M2 makers-positive (CD206+/CD163+) cells (3.8% vs 40%) (Fig 6A). This increase in M2 polarization was partially reversed when RenCa/RAW 264.7 co-culture was treated with BMP-6 or IL-10 neutralizing antibodies (Fig 6A). When RAW 264.7 and hPBMC were treated directly with 100 ng/ml of BMP-6 for 3 days, there was a 4-fold increase in M2 cells (0.8% vs 3.8% for RAW 264.7 and 3.8% vs 16.7% for hPBMC) (Fig 6B). The induction of M2 polarization by BMP-6 was again partially inhibited by anti-IL-10 antibody.

**Adoptive transfer of macrophages**

Although BMP-6 activates macrophages and induces the expression of type 1 proinflammatory cytokines including IL-6 and IL-1β (29, 34). Results of the present study suggest that the immunosuppressive effect of IL-10 appears to overcome the pro-inflammatory effects of IL-6 and IL-1β and mediates the overriding biological effect of BMP-6 that results in tumor growth in RCC. Therefore the disruption of IL-10 production by macrophages in the context of BMP-6 may tip the balance of local immune response in favor of inflammation that may lead to the activation of host immune system and tumor regression. To investigate this possibility, we again utilized the RenCa/BMP-6 tumor model in Balb/c mice following macrophage depletion with intraperitoneal injection of clodronate-liposome (Fig 6C). When wild type macrophages were adoptively transferred intratumorally, the induction of BMP-6 led to a dramatic increase in tumor growth. In contrast, the injection of IL-10KO macrophages not only blocked the pro-tumorigenic effect of BMP-6 but also decreased significantly the rate of tumor growth. The modest level of IL-10 detected in animals administered clodronate-liposome is likely due to the recovery of
native macrophages by the end of the experiment. Hematoxylin staining demonstrated a dramatic increase in neutrophil infiltration and necrosis in tumors injected with IL-10KO macrophages (Fig 6D).

Analysis in human clear cell RCC tissues

To determine the clinical relevance of the current findings, 124 human clear cell RCC tissues were examined. A typical example of positive immunohistochemistry for BMP-6, CD68, IL-10, and CD206 is shown in Fig 7A. Confocal immunofluorescence microscopy revealed that in samples that were BMP-6-positive, CD68, IL-10, and CD206 frequently co-localized (Fig 7B). When each marker was analyzed individually in the context of tumor stage, grade, and size, only CD68 and BMP-6 correlated with grade while BMP-6 was associated with stage (Supp Fig 5A). When cumulative survival rate was examined, higher tumor stage and grade were associated with poor prognosis (Supp Fig 5B and C); this confirms the validity of our samples. Subsequent analysis of the 4 markers failed to demonstrate any association with outcome (Supp Fig 5D). However, when each of the four markers were examined collectively or in various combinations, being positive for three markers - BMP-6, CD68, and IL-10 - was associated with decreased cumulative survival (Fig 7C); CD206 status did not add any further prognostic value. Next, a multivariate Cox analysis demonstrated that the three-marker signature (BMP-6/CD68/IL-10) was an independent predictor of cumulative survival (Supp Table 2). When ELISA was carried out with serum banked from 56 patients, BMP-6 levels were not high enough for detection. However, IL-10 serum level above 1 pg/ml was associated with an increased incidence of metastasis over a 5-year period (Fig 7D).

Taken together, we propose that clear cell RCC-derived BMP-6 stimulates the expression of the anti-inflammatory type 2 cytokine IL-10 in TAMs via a physical interaction between Smad5 and STAT3. IL-10, in turn, induces M2 polarization of TAMS and suppresses the local anti-tumor immune response (Fig 7E).

DISCUSSION

Because many types of solid tumors have elevated expression levels of BMPs, it has been proposed that BMPs may be oncogenic (35). Consistent with this hypothesis, we have observed that the overexpression of BMP-6 has a pro-tumorigenic effect in two murine RCC cell lines.
This oncogenic activity of BMP-6 though, was seen only in vivo, suggesting that BMP-6 stimulates RCC proliferation via a paracrine mechanism.

Common paracrine mechanisms of carcinogenesis include immune-modulation and angiogenesis. Results of the present study demonstrated that BMP-6 stimulates TAMs to produce IL-10, a type 2 cytokine that suppresses inflammation (36). The induction of IL-10 by BMP-6 was associated with M2 polarization of macrophages. This observation is consistent with the report that IL-10 induces M2 polarization of macrophages (14). Since M2 macrophages have poor antigen presenting capacity and suppress T cell activity (35), the pro-tumorigenic effect of BMP-6 is likely mediated by IL-10-induced M2 polarization of TAMs. Indeed, the removal of either macrophages or IL-10 reversed the pro-tumorigenic effect of BMP-6. In short, these results suggest that BMP-6 induces tumor growth by altering the tumor microenvironment in favor of anti-inflammation and local immune suppression. The possibility remains though that BMP-6 may also induce angiogenesis via a yet-to-be identified mechanism.

In the context of IL-10KO mice, BMP-6 induction resulted in a dramatic tumor regression. Since these mice developed no tumors when rechallenged, BMP-6 induces anti-tumor immune response in the absence of IL-10. Mechanistically, we have observed that BMP-6 also induces the expression of the pro-inflammatory type 1 cytokines IL-6 and IL-1β in macrophages (29, 34). Accordingly, in the absence of IL-10 acting as a counterweight, the balance of BMP-6’s effect in vivo may be skewed toward inflammation and anti-tumor response. Consistent with this concept, the adoptive transfer of macrophages from IL-10KO mice retarded BMP-6-induced tumor growth. Therefore, blocking IL-10 may be an effective therapy in a subgroup of RCC patients with dysregulated BMP-6/IL-10.

Canonical BMP signaling requires a heterotetrameric complex of types I and II receptors. To date, three each of types I and II receptors have been reported (37). Although these receptors are promiscuous, there is an optimal receptor combination for each BMP as it has been demonstrated that there are co-receptors that are relatively specific for BMP-2 and -4 (38). In the context of BMP-6/IL-10, BMP-RII along with ALK2 and 3 efficiently transduced BMP-6 signal. Whether there is a BMP-6-specific co-receptor remains to be studied.

Intracellularly, both Smad5 and STAT3 are necessary for IL-10 induction by BMP-6. ChIP demonstrated that both Smad5 and STAT3 bind to the same region within the IL-10 promoter. Since the IL-10 promoter contains STAT3 but not a consensus Smad binding element,
it is likely that STAT3 interacts directly with the DNA while Smad5 is a co-factor for the transcriptional activation of IL-10 by BMP-6. Further studies are necessary to verify this concept.

In clear cell RCC patients, BMP-6, CD68, IL-10, and CD206 had no prognostic value individually. This observation is consistent with the report that BMP-6 in clear cell RCC tissues does not predict outcome (12). Notwithstanding, patients who had the three-marker signature composed of BMP-6, CD68, and IL-10 were more likely to die from RCC. Interestingly though, CD206 (M2 macrophages) did not add any further prognostic value. This is likely due to colinearity as the presence of IL-10 and macrophages (CD68) were consistently associated with CD206. These observations suggest that BMP-6’s effect in RCC is dictated by the tumor microenvironment and that IL-10 may be a surrogate marker of BMP-6’s pro-tumorigenic effect.

Results of the present study implicate IL-10 as a new biomarker and a potential therapeutic target in RCC. Specifically, elevated serum levels of IL-10 were associated with increased risk of metastatic RCC. On the therapeutic front, an adoptive transfer of IL-10KO macrophages resulted in a dramatic tumor regression. Therefore, serum IL-10 levels may eventually be used to identify patients who are best candidates for targeting BMP-6/IL-10 loop. We are planning to test this concept in clear cell RCC patients using AS101, a small molecule inhibitor of IL-10 (39).

It should be pointed out that elevated tissue levels of IL-10 mRNA have been correlated with poor prognosis in RCC patients (40). Herein though, IL-10 alone did not predict outcome. The underlying reason for this difference is unclear. Possible explanations include the different methodologies used to detect IL-10 - immunohistochemistry vs RT-PCR. Further studies are needed to clarify this discrepancy.

In conclusion, BMP-6 is pro-tumorigenic in RCC. This oncogenic effect of BMP-6 is mediated by macrophage-derived IL-10 which leads to M2 polarization of TAMs. At the molecular level, BMP-6 induces IL-10 expression in macrophages via a direct interaction between STAT3 and Smad5 in the nucleus. In patients with clear cell RCC, the three-marker signature BMP-6/CD68/IL-10 predicted decreased cumulative survival after surgery while elevated serum IL-10 levels were associated with increased risk of metastasis. Taken together, these results demonstrate a novel mechanism of M2 polarization of TAMs in RCC that involves the BMP-6/IL-10 axis and provides the proof-of-principle for developing a personalized immunotherapy based on serum IL-10 level.
References


Figure Legends

Figure 1.

A. Representative screening using semi-quantitative RT-PCR for human BMP-6 expression in RenCa is shown. Clone #2 was selected for subsequent experiments.

B. ELISA for BMP-6 levels. Upto 120 pg/ml of BMP-6 was detected when cells treated with tetracycline.

C. MTT assay demonstrated that the induction of BMP-6 expression (Tet+) had no significant effect on the rate of cellular proliferation in vitro.

D. When RenCa/BMP-6 was inoculated subcutaneously into wild type mice and BMP-6 expression was induced (doxy+), the tumor growth rate increased significantly. Hematoxylin staining demonstrated no significant differences between doxy(-) and doxy(+) tumors. Induction of BMP-6 was confirmed by a RT-PCR and immunoblot analysis.

E. When BMP-6 expression was induced in mice that were administered clodronate (CL/Doxy+), the increased tumor growth rate observed in the control group (PBS/Doxy+) was completely blocked. PBS = Phosphate Buffered Saline; CL = clodronate. Hematoxylin staining demonstrated no significant change in histopathology macrophages were depleted.

Figure 2.

A. BMP-6 induced IL-10 mRNA (top panel) and protein (bottom panel) in a concentration-dependent manner in RAW 264.7, murine peritoneal macrophages (PMφs), THP-1 human monocyte cell line, and human peripheral blood macrophages (hPBMC). Lipopolysaccharide (LPS) was used as a positive control.

B. BMP-6 (100 ng/ml) induced IL-10 mRNA (top panel) and protein (bottom panel) expression in a time-dependent manner in RAW 264.7, PMφ, THP-1, and hPBMC.

C. When RenCa/BMP-6 was inoculated subcutaneously into IL-10KO mice, the induction of BMP-6 (doxy+) led to a dramatic regression of tumors and by 4 weeks, the tumors were no longer palpable.

D. RenCa/BMP-6 tumors were harvested prior to complete tumor regression. Hematoxylin staining demonstrated significant necrosis and neutrophil infiltration in tumors harvested from IL-10KO mice overexpressing BMP-6 (Doxy+).
**Figure 3.**

A. Semi-quantitative RT-PCR demonstrated that Actinomycin D (ActD) but not cycloheximide (CHX) blocked the induction of IL-10 by BMP-6 in PMs and RAW 264.7. CTL = control.

B. Each of the three type II BMP receptors (ActRIIA, ActRIIB, and BMPRII) and IL10-Luc reporter plasmid were co-transfected into RAW264.7. When treated with BMP-6, only the transfection of BMP-RII led to a significant increase in luciferase activity.

C. Each of the three type II BMP receptors was knocked down using the shRNA approach. When IL10-Luc was co-tranfected, only the knockdown of BMP-RII blocked IL-10 induction by BMP-6.

D. Constitutively active type I receptors –CA-ALK2 and CA-ALK3 – were co-transfected along with IL10-Luc into RAW 264.7. Increased luciferase activity was seen with the expression of either CA-ALK2 or CA-ALK3.

E. When either ALK2 or ALK3 was knocked down using shRNA, IL-10 induction by BMP-6 was blocked.

**Figure 4.**

A. When RAW 264.7 was transfected with the dominant-negative Smad4 (Smad4DN), the BMP-6-induced IL10 mRNA induction was completely blocked.

B. Each of the three R-Smads (Smad1, 5, and 8) was co-transfected with IL10-Luc into RAW 264.7. When treated with BMP-6 (100 ng/ml), only Smad5 overexpression significantly increased IL-10 promoter activity.

C. Each of the three R-Smads was knocked down with shRNA in RAW264.7. When co-transfected with IL10-Luc reporter and treated with BMP-6 (100 ng/ml), shRNA targeting Smad5 was the most efficient blocker of BMP-6-induced IL-10 promoter activity.

D. Promoter deletion analysis demonstrated that the most significant decrease in the BMP-6-induced IL-10 promoter activity was observed when the region between -800 to -588 bp 5’ upstream of the transcription start site was deleted.

E. ChIP assay demonstrated that Smad5 bound to the -800 to -588 bp region of IL-10 promoter in a ligand-dependent manner. As a control, exon 3 of IL-10 gene was amplified.

**Figure 5.**
A. RAW 264.7 was treated with BMP-6 (100 ng/ml) and immunoblot was carried out. The results demonstrated a dramatic increase in phosphorylated STAT3 within 15 min (0.25 hr) of BMP-6 treatment.

B. Inhibition of STAT3 with WP1066 (10 μg/ml) showed the abrogation of IL-10 induction by BMP-6 in RAW 264.7. WP1066 did not inhibit the LPS-induced IL-10 expression.

C. Transfection of dominant-negative STAT3 (STAT3DN) or siRNA targeting STAT3 blocked the induction of IL-10 by BMP-6 in RAW 264.7. The knockdown of STAT4 (siSTAT4) had no significant effect on IL-10 mRNA.

D. When RAW 264.7 was co-transfected with either STAT3 or STAT4 and IL10-Luc reporter and treated with BMP-6 (100 ng/ml), the overexpression of STAT3 significantly increased IL-10 promoter activity. Interestingly, STAT4 transfection repeatedly blocked the activation of IL-10 promoter.

E. ChIP assay demonstrated that STAT3 bound to the BMP-6 responsive region (-800 to -588 bp) within the IL-10 promoter in RAW 264.7. As control, exon 3 of IL-10 gene was amplified.

F. Immunoprecipitation against STAT3 pulled down Smad5. Conversely, immunoprecipitation against Smad5 pulled down STAT3.

G. Immunoprecipitation was carried out in cytosolic and nuclear fractions from RAW 264.7. Co-immunoprecipitation of Smad5 and STAT3 was observed in the nuclear fraction. The low level of STAT3 immunoprecipitated out with Smad5 in the nucleus in the absence of BMP-6 stimulation likely reflects the basal leakage of BMP-6.

H. In RenCa/BMP-6 tumors, there was a significant increase in phosphorylated levels of STAT3 and Smad5 in samples obtained from mice with BMP-6 induction (Doxy+).

I. Confocal immunofluorescence microscopy revealed that both pSTAT3 and pSmad1/5 co-localized to macrophages (F4/80+), especially in tumors harvested from Doxy(+) mice.

**Figure 6.**

A. Flow cytometry demonstrated M2 polarization (CD206+ or CD163+) when RAW 264.7 was co-cultured with RenCa. This M2 polarization was reversed partially when the cells were treated with neutralizing antibodies to either BMP-6 or IL-10.
B. RAW 264.7 and hPBMC were treated with BMP-6 (100 ng/ml). Flow cytometry again demonstrated a dramatic increase in M2 polarization of both cell types. This increase in M2 fraction induced by BMP-6 was blocked partially by IL-10 neutralizing antibodies.

C. Endogenous TAMs were depleted with clodronate-liposomes in forty Balb/c mice. When wild type macrophages (WT M) were injected into the tumors, induction of BMP-6 (WT/doxy+) resulted in a dramatic increase in tumor growth rate when compared to the control group (WT/doxy-). In contrast, the adoptive transfer of macrophages derived from IL-10KO mice led to a significant decrease in tumor growth with or without BMP-6 induction; however, BMP-6 induction resulted in the most significant decrease in tumor growth rate. RT-PCR demonstrated a decreased IL-10 mRNA level in the IL-10KO macrophages group (IL-10KO M). The low level of IL-10 expression detected in the IL-10KOM/Doxy+ group reflects the repopulation of tumors by endogenous macrophages as clodronate-liposome was injected only once at the beginning of the experiment.

D. Hematoxylin staining demonstrated an increase in neutrophil infiltration and tumor necrosis when IL-10KO Ms were adoptively transferred into tumors overexpressing BMP-6.

**Figure 7.**

A. Examples of human clear cell RCC specimens that were positive by immunohistochemistry for BMP-6, IL-10, macrophages (CD68+), and M2 polarization (CD206) are shown.

B. Confocal immunofluorescence microscopy was used to co-localize BMP-6, IL-10, macrophages (CD68+), and M2 polarization (CD206+) in human RCC tissues. BMP-6 and IL-10 frequently co-localized with CD68.

C. The prognostic value of BMP-6, CD68, IL-10, and CD206 was studied in 124 patients with clear cell RCC using Kaplan-Meier analysis. Having the three-marker signature (BMP-6/CD68/IL-10) was associated with a decreased cumulative survival.

D. Serum levels of IL-10 were correlated with the rate of 5-year metastasis in 56 patients with clear cell RCC. In patients with IL-10 serum levels above 1 pg/ml, there was a significantly higher rate of metastasis (p = 0.022).

E. Proposed model for BMP-6’s pro-tumorigenic effect in RCC. BMP-6 produced by tumor activates TAMs and induces the type 2 cytokine IL-10 expression via Smad5 and STAT3
interaction. IL-10, then, induces M2 polarization of TAMs and suppresses the local anti-tumor immune response, thus leading to tumor proliferation and progression.
Figure 1
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A. Tet
BMP-6
β-Actin

B. BMP-6 concentration (pg/ml)

C. % growth

D. Doxy

E. Tumor size (%)

Research.
Figure 2
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A. BMP6 (ng/ml) 0 1 10 100 LPS 0 1 10 100 LPS 0 1 10 100 LPS 0 1 10 100 LPS
IL-10
β-Actin
IL-10
β-Actin
RAW 264.7 PMφ THP-1 hPBMC

B. Time (hr) 0 1 6 12 24 0 1 6 12 24 0 1 6 12 24 0 1 6 12 24
IL-10
β-Actin
IL-10
β-Actin
RAW 264.7 PMφ THP-1 hPBMC

C. Tumor size (mm³)

D. Doxy
BMP-6
IL-10
β-Actin
Doxy
- - - +

Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

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Figure 3
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A. BMP6 +
IL-10 - +
β-Actin - +
BMP6 +
IL-10 - +
β-Actin - +

D. % Luciferase Activity
Control CA-ALK2 CA-ALK3

B. % Luciferase Activity
Control ActRlla ActRlrb BMPRII

E. % Luciferase Activity
CTL SH-ALK2 SH-ALK3

C. % Luciferase activity
CTL Sh-ActRlla Sh-ActRlrb Sh-BMPRII
Figure 4
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A. BMP6 (100ng) Vector - + Smad4DN - + IL-10 - + β-Actin - +

B. BMP6(-) □ BMP6(+) ■

C. BMP-6 (-) □ BMP-6 (+) ■

D. -714/-700 STAT -233/-237 CCAAT -91/-88 TATA

E. BMP6 IL10 -800 -588

Control

sh-Smad1

sh-Smad5

sh-Smad8

sh-Smad4
Figure 5
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A. Time (hrs) 0 0.25 0.5 1 6 12 24
pSTAT3
Total STAT3

IL-10
β-actin
BMP6 - + - + - -
WP1066 - - + - + +
LPS - - - - + +
Vector STAT3DN
BMP6 (100ng) - + - + +
IL-10
β-Act
siSTAT4 siSTAT3
BMP6 (100ng) - + - + +

BMP-6
IL10 -800 -588
Control

C. Input: STAT3
Input: Smad5
Input: β-actin

G. C N C N
IP: Smad5
WB: STAT3
IP: STAT3
WB: Smad5
Input: Smad5
Input: STAT3
Input: Ki67
Input: GAPDH

D. % luciferase activity

Doxy Doxy
- + - +
P-STAT3 P-SMAD1/5
Total STAT3 SMAD1/5
BMP-6 BMP-6
β-actin β-actin

E. BMP6 - +
IL10 -800 -588
Control

F. BMP-6 - +
IP : STAT3
IB : Smad5
IP : Smad5
IB : STAT3
IP : Smad5
IB : STAT3
Input : STAT3
Input : Smad5
Input : β-actin

H. Doxy Doxy
- + - +
P-STAT3 P-SMAD1/5
Total STAT3 SMAD1/5
BMP-6 BMP-6
β-actin β-actin

I. p-STAT3 pSmad 1/5 F4/80 (Mφ) merge

Doxy -

Doxy +
Figure 6

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A. **Table Description**

B. **Graph Description**

C. **Graph Description**

D. **Image Description**
Figure 7
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A. BMP-6  IL-10  CD68  CD206

B. BMP6  CD68  IL10  Merge

CD68  IL-10  CD206  Merge

C. BMP-6/CD68/IL-10 (+/+/+)
P < 0.001

D. 5-yr metastasis

No Met  Met

Serum IL-10

< 1 pg/ml  ≥ 1 pg/ml

E. ALK2/3 BMPRII Smad5 STAT3 Smad5 IL-10

RCC

TAM

Tumor proliferation

BMP-6

CD68

IL-10

CD206

C. Cumulative Survival

BMP-6/CD68/IL-10 (+/+/+)

P < 0.001